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KIM MARSHALL
MANAGER EXAMINATION SUPPORT AND SALES

P/00/009 28/5/91
Regulation 3.2

AUSTRALIA

Patents Act 1990

ORIGINAL

PROVISIONAL SPECIFICATION

VECTOR

The invention is described in the following statement:

VECTOR

FIELD OF INVENTION

This invention relates to delivery vectors for antigen producing genes (heterologous gene sequences) used to generate immune responses in commercial pigs susceptible to decimation by disease. Such vectors are especially useful for the preparation of vaccines which can be easily administered on a large scale to protect pigs against disease. This invention also relates to a method of production of suitable delivery vectors, to methods of preparation of vaccines based on the vectors, to administration strategies and to a method protecting pigs from disease.

BACKGROUND

The productivity of the intensive pig industry depends on the control of infectious diseases. Whilst diseases can be controlled in part by good hygiene and quarantine measures, the industry must still rely on vaccination to protect herds. In a commercial situation, the cost per animal is high in terms of feed and current disease control costs and therefore, the costs in disease prevention and control by any newly proposed vaccine must be cheap, effective and easy to deliver.

Conventionally, vaccines constituting live viral particles have been prepared by virus passage and selection of attenuated forms. Alternatively, killed vaccines were prepared from virulent viruses.

The most recent description of the use of viral vectors in the control of disease in pigs was the deletion mutant of pseudorabies virus for the control of Aujesky's disease. The use of a herpesvirus as a vector has the advantage of being able to stimulate a humoral and cell-mediated response, thus providing possible life long protection. Another advantage is the ability to insert other heterologous sequences in this vector, being expressed from a suitable promoter, to produce antigens for exposure to the animals immune system, thus protecting against two diseases. There are disadvantages of this system. Firstly, there is the issue of latency. Herpesviruses have the ability to intergrate into the neurons in ganglia for the life of the animal. It only requires a suitable stress on the animal to cause the reactivation of the virus and consequently full

disease. However, it is now known that the deletion of a specific gene, glycoprotein E, will attenuate the virus and prevent reactivation from latency. Therefore, this deletion vector is now widely used as an eradication vector for Aujesky's disease and subsequently will not be available as a suitable vector for the delivery of other antigens.

It is thus the aim of this invention to provide a delivery vehicle for heterologous sequences of genetic material that is particularly suited to administration on a large scale.

In particular, it is the aim of this invention to provide or enhance means for generation and/or optimisation of antibodies or cell-mediated immunity so as to provide protection against infection with common porcine diseases. It is an additional aim to provide a process for preparation of a suitable means for generation and/or optimisation of antibodies or cell-mediated immunity so as to protect pigs against infection with common porcine diseases. It is a further aim to provide a protection strategy.

SUMMARY OF INVENTION

The invention provides, in one embodiment, a recombinant porcine adenovirus capable of expressing DNA of interest, said DNA of interest being stably integrated into an appropriate site of said recombinant porcine adenovirus genome.

In another embodiment the invention provides a recombinant vector comprising a recombinant porcine adenovirus which incorporates at least one heterologous nucleotide sequence. Preferably the heterologous nucleotide sequence is capable of expression as an antigenic polypeptide. The antigenic polypeptide encoded by at least one nucleotide sequence is preferably foreign to the host vector.

The recombinant vector may comprise a live recombinant porcine adenovirus in which the virion structural proteins are unchanged from those in the native porcine adenovirus from which the recombinant porcine adenovirus is produced.

This invention is partially predicated on the discovery that there are non-essential regions in the porcine adenovirus genome which do not correspond to

those characterised previously on other adenoviruses thus making this virus particularly suited to delivery of heterologous sequences.

This invention is also predicated on the discovery that the porcine adenovirus generates a prolonged response in pigs thus making it well suited as a vaccine vehicle. Furthermore, the existence of a number of serotypes specific to respiratory or gastrointestinal tracts, allows the selection of a vaccine vehicle suited to a target organ and the type of immune response required.

The invention is also predicated on the discovery that porcine adenovirus can package genomic DNA greater than the 105% rule for mammalian adenoviruses with intermediate size genomes and that the resultant packaged virions are stable in vitro and in vivo.

Adenoviruses are a large and diverse family, having been isolated from many living species, including man and other mammals as well as a variety of birds. As a result adenoviruses have been separated into two different genera, one group has a mammalian host range (*Mastadenoviridae*) and the other an avian host range (*Aviadenoviridae*).

Porcine adenoviruses are prevalent infectious agents of pigs and to date four distinct serotypes have been recognised (Adair and McFerran, 1976) and evidence for at least one more (Derbyshire *et al.*, 1975). Of the four serotypes found, three (serotypes 1 to 3) were isolated from the gastrointestinal tract while the fourth was recovered from the respiratory system. The porcine adenoviruses are considered to be a low pathogenic widespread agent and although isolations were made in general from diseased animals, it was most likely that the adenovirus was present only as a secondary infection. They have been isolated from pigs with diarrhoea and respiratory infections but it has been considered that at least the gastrointestinal adenovirus infections are usually asymptomatic (Sanford and Hoover, 1983). Porcine adenoviruses are spread by ingestion or inhalation and experimental infection via oral, intranasal and intratracheal inoculations have resulted in uptake of the virus. Experimental pathogenicity studies have shown that the primary sites of infection are the lower small intestine probably the tonsil (Sharpe and Jessett, 1967; Shadduck *et al.*, 1968). With serotype 4 infection, a viraemia appears to develop in

experimental infections. However, this may be a less common manifestation with the gastrointestinal serotypes (Shadduck *et al.*, 1968). Faecal excretion is the most common cause for spread of PAV, being present for several weeks post infection. Nasal shedding also occurs under experimental conditions. PAV's role in pneumonia has been suggested to be that of either a predisposing factor or a synergist (Kasza *et al.*, 1969; Schiefer *et al.*, 1974) but experimental pneumonia with serotype 4 did not require a second agent to produce disease (Smith *et al.*,1973).

Porcine adenoviruses have yet to be examined in much detail and little is known about their role in disease or how common they are. This is due to the fact that they do not produce any significant disease in herds and have failed to draw the interest of industry through loss of production. It is likely that the number of serotypes of porcine adenoviruses is much greater than four and that it probably exists in the majority of pig herds as a normal commensal.

Work done on porcine adenovirus in regards to its morphology and molecular biology, has shown some similarities with other *Mastadenoviruses* examined. Its morphology is that of other adenoviruses examined with an icosahedral capsid containing a core of a double stranded DNA genome. Very little work on the characterisation of the PAV genome has been published (Benkö *et al.*, 1990, Kleiboeker *et al.*, 1993, Reddy *et al.*, 1993, Kleiboeker, 1994). The size of the PAV genome (approx. 34.8 kb) is slightly smaller than that of human adenoviruses (approx. 35.9 kb). One study has shown using hybridisation with DNA probes from the total genome of human adenovirus type 2 that there is reasonable DNA homology between the porcine and human adenoviruses (Benkö *et al.*, 1990). A recent report on the serotype 4 PAV demonstrated that its genomic layout was also similar to that of the human adenoviruses in the area of the L4 and E3 regions (including the 33K and pVIII genes) even though the sequence homology was not as strong as may have been expected (Kleiboeker, 1994).

While choosing appropriate PAV for development as a live vectors to deliver vaccines to pigs, it is important to take into account the natural prevalence of serotypes. Those serotypes not commonly encountered in the

field have an obvious advantages over those to which pigs are frequently exposed and to which they may have developed immunity.

A further consideration is the ability of the vector to remain active in the pig beyond the period which maternal antibodies in colostrum protect pigs immediately post-birth.

Other important considerations in choosing potential PAV vectors are pathogenicity and immunogenicity. Preferably live vector viruses should be highly infectious but non-pathogenic (or at least attenuated) such that they do not themselves adversely affect the target species.

The preferred candidates for vaccine vectors are non-pathogenic isolates of serotype 4 (respiratory) and serotype 3 (gastrointestinal). Serotype 3 has been chosen as the serotype of choice due to excellent growth abilities in continuous pig kidney cell lines. The isolation of other serotypes, which seems likely, may well alter this selection. It is notable that the more virulent strains produce a greater antibody response.

Heterologous nucleotide sequences which may be incorporated into non-essential regions of the viral genome and which may encode the antigenic determinants of infectious organisms against which the generation of antibodies or cell-mediated immunity is desirable may be those expressing antigenic determinants of intestinal infections caused by gastrointestinal viruses; for example rotavirus or parvovirus infections, or respiratory viruses, for example parainfluenza virus, or that of Japanese encephalitis.

Heterologous nucleotide sequences which may be incorporated include the antigenic determinants of the agents of:

Porcine parvovirus	Mycoplasma hyopneumonia
Porcine parainfluenza	Transmissable gastroenteritis (porcine coronavirus)
Porcine rotavirus	Hog cholera virus (Classical swine fever)
Swine dysentery	African swine fever virus

Heterologous nucleotide sequences more preferred for incorporation in the vectors of the invention are those expressing antigenic determinants of porcine parvovirus, porcine rotavirus, porcine coronavirus and classical swine

fever virus.

It is also envisaged the heterologous sequences incorporated may be immuno-potentiator molecules such as cytokines or growth promoters, for example porcine interleukin 4 (IL4), gamma interferon (γIFN) and granulocyte macrophage colony stimulating factor (GM-CSF).

The type of immune response stimulated by candidate vectors may affect the selection of heterologous nucleotide sequences for insertion therein. PAV serotypes 1, 2 and 3, which naturally infect via the gut may induce local mucosal immunity and are thus more suitable for infections of the intestines (eg classical swine fever virus). PAV serotype 4, which naturally infects via the respiratory system, may be more suitable for infections of the respiratory tract (eg porcine parainfluenza) may also induce good local immunity.

The DNA of interest which may comprise heterologous genes coding for antigenic determinants or immuno-potentiator molecules may be located in at least one non-essential region of the viral genome.

Non-essential regions of the viral genome which may be suitable for the purposes of replacement with or insertion of heterologous nucleotide sequences may be non-coding regions at the right terminal end of the genome at map units 97 to 99.5.

The heterologous gene sequences may be associated with a promoter and leader sequence in order that the nucleotide sequence may be expressed in situ as efficiently as possible. Preferably the heterologous gene sequence is associated with the porcine adenoviral major late promoter and splice leader sequence. The mammalian adenovirus major late promoter lies near 16-17 map units on the adenovirus genetic map and contains a classical TATA sequence motif (Johnson, D.C., Ghosh-Chondhury, G., Smiley, J.R., Fallis, L. and Graham, F.L. (1988), Abundant expression of herpes simplex virus glycoprotein gB using an adenovirus vector. Virology 164, 1-14).

The splice leader sequence of the porcine adenovirus serotype under consideration is a tripartite sequence spliced to the 5' end of the mRNA of all late genes.

The heterologous gene sequence may also be associated with a poly

adenylation sequence.

Instead of the porcine adenoviral major late promoter, any other suitable eukaryotic promoter may be used. For example, those of SV40 virus, cytomegalovirus (CMV) or human adenovirus may be used.

Processing and poly adenylation signals other than those of porcine adenoviruses may also be considered, for example, that of SV40.

In a further aspect of the invention there is provided a recombinant vaccine for generating and/or optimising antibodies or cell-mediated immunity so as to provide or enhance protection against infection with an infectious organism in pigs, the vaccine comprising at least one recombinant porcine adenovirus vector incorporating at least one heterologous nucleotide sequence formulated with suitable carriers and excipients. Preferably the nucleotide sequence is capable of expression as an antigenic polypeptide.

The antigenic polypeptide encoded by the at least one nucleotide sequence is preferably foreign to the host vector. At least one nucleotide sequence may be associated with a promoter/leader and a poly A sequence.

The recombinant vaccine may include live recombinant porcine adenovirus vector in which the virion structural proteins are unchanged from that in the native porcine adenovirus from which the recombinant porcine adenovirus is produced.

Preferred vector candidates for use in the recombinant vaccine are PAV isolates of serotype 3 and 4. Use of other serotypes is possible, depending on herd existing immunity and its environment.

The vaccine may be directed against respiratory and intestinal infections caused by a variety of agents. In order to direct the vaccine against a specific infectious organism, heterologous gene sequences encoding the antigenic determinants of those infectious organisms may be incorporated into non-essential regions of the genome of the porcine adenovirus comprising the vector. If the vaccine is to be used to optimise protection against disease, suitable heterologous nucleotide sequences may be those of immuno-potentiators such as cytokines or growth promoters.

The vaccine may comprise other constituents, such as stabilisers,

excipients, other pharmaceutically acceptable compounds or any other antigen or part thereof. The vaccine may be in the form of a lyophilised preparation or as a suspension, all of which are common in the field of vaccine production.

A suitable carrier for such as a vaccine may be isotonic buffered saline.

In a further aspect of the invention, there is provided a method of preparing a vaccine for generation and/or optimisation of antibodies or cell-mediated immunity so as to induced or enhance protection against an infectious organism in a pig, which comprises constructing a recombinant porcine adenovirus vector incorporating at least one heterologous nucleotide sequence, and placing said recombinant porcine adenovirus vector in a form suitable for administration. Preferably the nucleotide sequence is capable of expression as an antigenic polypeptide although it may also be an immuno-potentiator. The nucleotide sequence is conveniently foreign to the host vector.

More preferably the nucleotide sequence is associated with promoter/leader and poly A sequences.

The form of administration may be that of an enteric coated dosage unit, an inoculum for intra-peritoneal, intramuscular or subcutaneous administration, an aerosol spray, by oral or intranasal application. Administration in the drinking water or in feed pellets is also possible.

In another aspect of the invention, there is provided a method of producing a porcine adenovirus vaccine vector which comprises inserting into a porcine adenovirus at least one heterologous nucleotide sequence. Said heterologous nucleotide sequence is preferably capable of expression as an antigenic polypeptide.

Preferably the antigenic polypeptide encoded by the at least one nucleotide sequence is foreign to the host vector.

More preferably the heterologous nucleotide sequence is associated with promoter/leader and poly A sequences.

In one method of construction of a suitable vector the non-essential region to be altered to incorporate foreign DNA could be constructed via homologous recombination. By this method the non-essential region is cloned and foreign DNA together with promoter, leader and poly adenylation

sequences is inserted preferably by homologous recombination between flanking sequences. By this method also, deletion of portions of the non-essential region is possible to create extra room for larger DNA inserts that are beyond the normal packing constraints of the virus.

By this method a DNA expression cassette containing an appropriate PAV promoter with foreign gene sequence as well as leader sequences and poly adenylation recognition sequences can be constructed with the unique restriction enzyme sites flanking the cassette enabling easy insertion into the PAV genome.

In another aspect of the invention there is provided strategies for administration of the vaccines of the invention.

In one strategy, a heterologous antigen and immuno-modulatory molecule such as a cytokine may be expressed in the same recombinant and delivered as a single vaccine.

In one strategy according to the invention PAV vector based vaccines may be administered as 'cocktails' comprising 2 or more virus vectors carrying different foreign genes or immuno-potentiators.

In a preferred vaccination strategy of the invention, the 'cocktail' or simultaneous strategy, a vaccine based on both PAV serotype 3 and serotype 4 is used.

In another preferred strategy, a base recombinant serotype 3 porcine adenovirus is constructed and the fiber gene from serotype 4 replacing that of serotype 3 or the fiber from serotype 4 additionally cloned into the vaccine to broaden the targeting of the invention to both gut and respiratory delivery.

In an alternative strategy according to the invention, PAV vector based vaccines may be administered consecutively of each other to either administer booster vaccines or new vaccines at some stage subsequent to initial PAV vaccination. The vaccines used are preferably based on heterologous PAV isolates.

In a preferred version of the "consecutive" strategy, vaccines based on isolates serotypically unrelated are selected so as to achieve maximum protection against infection. In one example of such a strategy a vaccine based

on PAV serotype 3 is administered subsequently or prior to vaccination with a vaccine based on PAV serotype 4.

Pigs are conveniently inoculated with vector vaccines according to the invention at any age. Piglets may be vaccinated at 1 day old, breeders may be vaccinated regularly up to point of giving birth and thereafter.

Preferably according to either the consecutive strategy or the cocktail strategy, pigs are vaccinated while still not fully immunocompetent. More conveniently, day-old pigs can be vaccinated for protection against re-infection after a period of 4 weeks subsequent to initial vaccination.

In a further embodiment of the invention there is provided a method for producing an immune response in a pig comprising administering to the pig an effective amount of a recombinant vaccine according to the invention. An effective amount is an amount sufficient to elicit an immune response, preferably at least 10^4 $TCID_{50}$ per dose.

The vaccine of the invention may of course be combined with vaccines against other viruses or organisms such as parvovirus or Aujeszky's disease at the time of its administration.

In a preferred aspect of this embodiment of the invention, administration is by oral delivery or intra-nasally.

Methods for construction and testing of recombinant vectors and vaccines according to this invention will be well known to those skilled in the art. Standard procedures for endonuclease digestion, ligation and electrophoresis were carried out in accordance with the manufacturer's or suppliers instructions. Standard techniques are not described in detail and will be well understood by persons skilled in the art.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates the DNA restriction endonuclease map of the entire PAV genome.

Figure 2 illustrates the sequence characterisation and cloning of the major later promoter and splice leader sequences of PAV serotype 3.

Figure 3 illustrates the sequences of the major later promoter, upstream enhancer sequence and splice leaders 1, 2 and 3.

Figure 4 illustrates the terminal 720 basis of the right end of the genome.

Figure 5 illustrates the promoter region of E3 and the overlapping L4 area.

Figure 6 illustrates a preferred method of construction of a PAV vector.

Figure 7 tabulates the results of animal trials using a PAV based vaccine as set out in example 2.

Figure 8 graphically represents the results of figure 7.

PREFERRED EMBODIMENTS

Aspects of preferred embodiments of the invention based on PAV isolates serotype 3 and serotype 4 will now be described. Whilst these two isolates have been selected because of their sites of infection in the pig, it will be appreciated that other isolates of porcine adenovirus may be more suitable for construction of vaccine vectors provided the criteria for selection described herein before are met.

In general, PAV are considered of low pathogenicity with little consequence in the field. The pathogenic significance of PAV is reviewed in Derbyshire, 1989. The first report of isolation of PAV was from a 12 day old pig with diarrhoea (Haig *et al.*, 1964). Two years later, PAV type 4 was first reported, isolated from the brain of a pig suffering from encephalitis of unknown cause (Kasza, 1966). Later reports have associated PAV mainly with diarrhoea in the field although this is normally low grade. PAV can also be regularly isolated from healthy animals with no disease signs and it is quite likely that its isolation from diseased animals is more a coincidence of its prevalence than an indicator of pathogenicity. However, an association between serotype 4 and respiratory disease has been reported (Watt, 1978) and this has been supported by experimental infection (Edington *et al.*, 1972). Experimental infections with gastrointestinal serotypes of the virus (eg serotype 3) have been able to produce diarrhoea but the pathological changes produced were not clinically significant.

The genome of the selected PAV serotype 3 was characterised by conventional methods. The DNA restriction endonuclease maps of the entire genome is illustrated in figure 1. The genomes are orientated left to right. By

convention adenovirus genomes are normally orientated such that the terminal region from which no late mRNA transcripts are synthesised is located at the left end. The enzymes used to generate the map are indicated at the edge of each map.

CHARACTERISATION OF MAJOR LATE PROMOTER (MLP) AND SPLICE LEADER SEQUENCES (LS) OF PAV SEROTYPE 3

Identification and cloning of the PAV MLP

By use of restriction enzyme and genetic maps of the PAV serotype 3 genome, a region was located that contained the MLP and leader sequences (Fig 1). The fragments identified in this region were cloned into plasmid vectors and sequenced.

The MLP promoter sequence was identified as containing a classical TATA sequence, the only one in the region sequenced, as well as upstream factors and was subsequently confirmed by the location of the leader sequence and the transcriptional start site.

Figure 2 illustrates the sequence characterisation and cloning of the major late promoter and splice leader sequences of PAV serotype 3. Specifically shown are the *Apa*I and *Xho*I restriction endonuclease maps of PAV serotype 3 (Fig 1).

In order to determine the structure and sequence of the leader sequence spliced to late mRNA, porcine kidney cells were infected with PAV and the infection was allowed to proceed until late in the infection cycle (usually 20-24 hr p.i.). At this time total RNA was purified from the infected cells using the RNAgents total RNA purification kit (Promega). The isolated RNA was precipitated with isopropanol and stored at -70°C in 200 µl aliquots until required. Poly A (mRNA) was isolated from total RNA by the use of the Poly AT tract System (Promega, USA). The isolated mRNA was used in cDNA production.

For cDNA production, oligonucleotides were produced to the complimentary strand of the hexon gene and the penton base gene, both being MLP transcripts. A further oligonucleotide was produced which covered the proposed cap site of the major late transcript, 24 bases downstream of the TATA

box. This oligonucleotide was used in conjunction with that used in cDNA production in Taq polymerase chain reaction. The resulting DNA produced from positive clones was digested with appropriate restriction enzymes to determine the size of the inserted fragment. DNA sequencing of these inserted fragments was performed using a modification of the chain termination technique (Sanger, F., Nicklen, S and Gulson, A.R., 1977, DNA sequencing with chain terminating inhibitors. PNAS USA 74: 5463-5467) so as to allow Taq DNA polymerase extension (Promega, USA).

To confirm the leader sequence cap site, fresh cDNA was prepared and this time a tail of dGTP residues added to it. Briefly, cDNA was incubated with 1 mM dGTP and approximately 15 units of terminal deoxynucleotidyl transferase (Promega) in 2 mM CaCl2 buffer at 37°C for 60 minutes. The reaction was stopped by heating to 70°C for 10 minutes. The DNA was then ethanol precipitated and resuspended in a volume suitable for use in polymerase chain reaction (PCR). PCR was performed as previously described using a poly (dC) oligonucleotide with a *Xba*I site at the 5' end. Resulting fragments were blunt ended with T4 DNA polymerase at 37°C for 30 minutes in the presence of excess nucleotides and cloned into the *Sma*I site of the pUC18 vector. DNA preparation and sequencing were performed, as described previously, on clones shown to be positive by hybridisation.

Figure 3 illustrates the separate sequences of the major late promoter, upstream enhancer sequence and splice leaders 1, 2 and 3 as determined from cDNA studies. Figure 2 illustrates the DNA sequence of the complete promoter cassette with the components joined together.

CHARACTERISATION OF NON-ESSENTIAL REGIONS OF VIRAL GENOME

The right end was identified by cloning and complete sequencing of the PAV serotype 3 *Apa*I fragment J of approximately 1.8 Kbp. The inverted terminal repeat (ITR) has been determined by comparison of the RHE sequence with that of the left hand end. The ITR is 144 bases long and represents the starting point into which potential insertions can be made. Figure 4 shows the sequence of the terminal 720 bases. Restriction endonuclease sites of interest for insertion of foreign DNA are indicated in the terminal sequence. A putative

TATA site for the E4 promoter is identified, this being the left most end for the possible site of insertion. Initial insertions will be made into the *Sma*I or *Eco*RI sites.

The E3 region of the genome, this also being a non-essential area, has been located and cloned. The promoter region of E3 has been identified and the overlapping L4 area sequenced (Figure 5). The region of the E3 after the polyadenylation signal of the L4 is also a possible site for insertion and can also be used for deletion to create more room for larger cassette insertions.

CONSTRUCTION OF PAV VECTOR

Figure 6 illustrates a preferred method of construction of a PAV vector. The right hand end *Apa*I fragment J of PAV serotype 3 is cloned and a unique *Sma*I restriction endonuclease site 230 bp from the inverted repeats was used as an insertion site.

The major late promoter expression cassette containing the E2 (gp55) gene of classical swine fever (hog cholera virus) was cloned into the *Sma*I site of the RHE fragment.

A preferred method of homologous recombination was cutting genomic PAV 3 DNA with *Hpa*I, a unique site in the genome, and transfecting this DNA with *Apa*I cut expression cassette plasmid containing gp55.

The DNA mix was transfected into preferably primary pig kidney cells by standard calcium chloride precipitation techniques.

The preferred method of transfection generates recombinant virus through homologous recombination between genomic PAV 3 and plasmid (Fig 6).

DETAILED DESCRIPTION OF THE INVENTION

The following example show the construction of a representative recombinant porcine adenovirus of this invention. The recombinant virus was propagated and titred on primary porcine kidney cells.

Example 1.

An expression cassette consisting of the porcine adenovirus major late promoter, the classical swine fever virus (CSFV) gp55 and SV 40 polyA was inserted into the *Sma*I site of the right hand end (MU 97-99.5) of porcine

adenovirus serotype 3 and used to generate in porcine primary kidney cells a recombinant PAV 3. The size of the expression cassette was 2.38 kilobase pairs. No deletion of the genomic PAV 3 was made. Mammalian adenoviruses with intermediate genomes (~36kb) have been shown to accommodate up to 105% of the wild-type genomic length, and genomes larger than this size are either unpackageable or extremely unstable, frequently undergoing DNA rearrangements (Betts, Prevec and Graham, Journal of Virology 67, 5911-5921 (1993), Packaging capacity and stability of human adenovirus type 5 vectors: Parks and Graham, Journal of Virology, 71, 3293-3298, (1997), A helper dependent system for adenovirus vector production helps define a lower limit for efficient DNA packaging). In this invention, PAV genomic length was 34.8 kb, into which was inserted without any other deletion an expression cassette of 2.38 kb. The resulting genomic DNA length of the recombinant porcine adenovirus of this invention was 106.8%, and therefore exceeded the maximum limit for construction of a stable recombinant. The recombinant virus was plaque purified three times and passaged stably in primary pig kidney cells. The recombinant was shown to contain gp55 by Southern blot hybridisation. Expression of gp55 was demonstrated by infecting primary PK cell line grown on glass cover slips with the recombinant porcine adenovirus. After 24 hours, immunoflouresencent staining (IF) showed infected cells expressing gp55.

Example 2.

Immunisation of pigs with recombinant PAV::gp55

Out of three piglets (5-6 weeks of age), one (#4) was infected with recombinant PAV::gp55 (plaque #21) by subcutaneous route and two others (#16, #24) were left unvaccinated. For infection 1 X 10^7 pfu of PAV is applied to each animal.

No clinical signs were observed with no rise in temperature.

Challenge with classical swine fever

Four weeks after immunisation with the recombinant PAV, the pigs were challenged with 1 X $10^{3.5}$ $TCID_{50}$ classical swine fever virus "Weybridge". Virus was applied subcutaneous. This amount of virus has been experimentally determined to be lethal for pigs.

On day 3 after challenge all pigs had temperatures between 40.5 and 41.5 °C. At day 5 the control pigs (#16, #24) were greater than 41°C. The control pigs maintained greater than 41°C until terminated at days 7 and 8. Pig #4 revealed fever until day 5, then declined in temperature to below 40°C at days 10 and 11. Control pigs (#16,#24) were dead or euthanased by day 8. Therefore, one pig (#4) vaccinated subcutaneously with the recombinant PAV returned to normal temperature and survived 3 days longer than all other pigs. These results are tabulated in figure 7, and graphically represented by figure 8.

It will be appreciated that whilst this document establishes the metes and bounds of this invention, all embodiments falling within its scope for example with regard to heterologous genes, insertion sites, types of promoter and serotype have not necessarily been specifically exemplified although it is intended that they should fall within the scope of protection afforded this invention.

DATED this 13th day of August, 1997.

COMMONWEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANISATION

WATERMARK PATENT & TRADEMARK ATTORNEYS
290 BURWOOD ROAD
HAWTHORN VICTORIA 3122
AUSTRALIA

VAX DOC 16 AU002374.WPC KJS:se

Restriction enzyme maps of the PAV3 genome

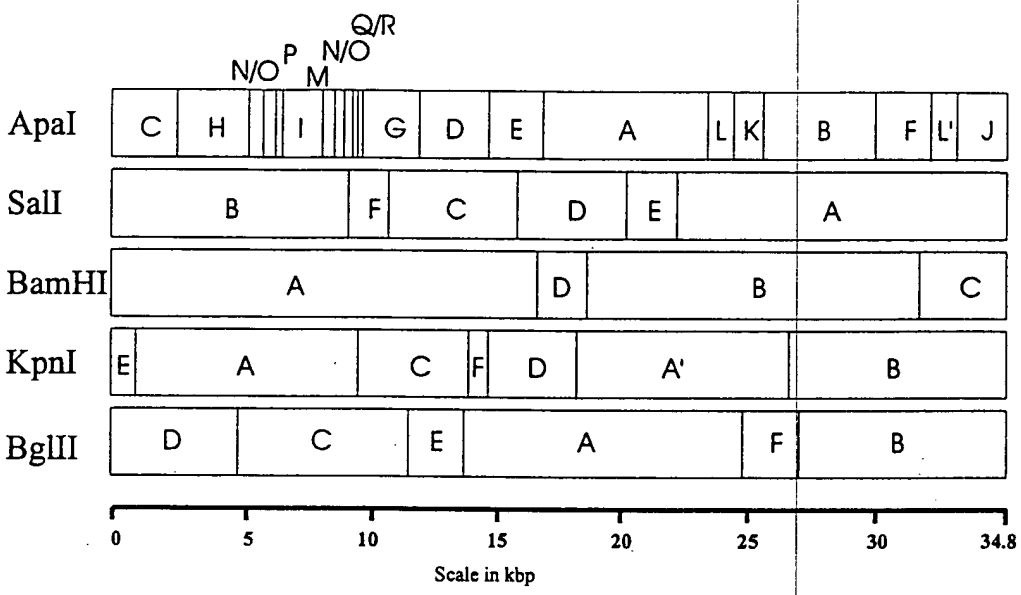


Figure 1.

Figure 2

Total sequence of the PAV Major Late Promoter cassette including the added nucleotides 5' (upstream) of the USF.

Nucleotide base count: 76 A 143 C 187 G 96 T Total 502 bp

```
  1 GGTGCCGCGG TCGTCGGCGT AGAGGATGAG GGCCCAGTCG GAGATGAAGG CACGCGCCCA
 61 GGCGAGGACG AAGCTGGCGA CCTGCGAGGG GTAGCGGTCG TTGGGCACTA ATGGCGAGGC
121 CTGCTCGAGC GTGTGGAGAC AGAGGTCCTC GTCGTCCGCG TCCAGGAAGT GGATTGGTCG
181 CCAGTGGTAG TCCACGTGAC CGGCTTGCGG GTCGGGGGGT ATAAAAGGCG CGGGCCGGGG
241 TGCGTGGCCG TCAGTTGCTT CGCAGGCCTC GTCACCGGAG TCCGCGTCTC CGGCGTCTCG
301 CGCTGCGGCT GCATCTGTGG TCCCGGAGTC TTCAGGTCCT TGTTGAGGAG GTACTCCTGA
361 TCGCTGTCCC AGTACTTGGC GTGTGGGAAG CCGTCCTGAT CGCGATCCTC CTGCTGTTGC
421 AGCGCTTCGG CAAACACGCG CACCTGCTCT TCGGACCCGG CGAAGCGTTC GACGAAGGCG
481 TCTAGCCAGC AACAGTCGCA AG
```

The Upstream Stimulatory Factor (USF) and TATA motiff are in bold. The complete leader sequence is italised with the cap site and splice sites between the individual leaders indicated by double underlining or single underlining respectively.

Figure 3.

Individual sequences of the Promoter cassette components:

I. The 5' (upstream) sequence included in the long cassette.

```
  1 GGTGCCGCGG TCGTCGGCGT AGAGGATGAG GGCCCAGTCG GAGATGAAGG CACGCGCCCA
 61 GGCGAGGACG AAGCTGGCGA CCTGCGAGGG GTAGCGGTCG TTGGGCACTA ATGGCGAGGC
121 CTGCTCGAGC GTGTGGAGAC AGAGGTCCTC GTCGTCCGCG TCCAGGAAGT GGATTGGTCG
181 CCAGTGGTAG
```

II. Sequence including the USF, TATA motiff and sequence to the cap site.

```
  1 CCACGTGACC GGCTTGCGGG TCGGGGGGTA TAAAAGGCGC GGGCCGGGGT GCGTGGCCGT
 61 C
```

III. First leader sequence.

```
  1 AGTTGCTTCG CAGGCCTCGT CACCGGAGTC CGCGTCTCCG GCGTCTCGCG CTGCGGCTGC
 61 ATCTGTGGTC CCGGAGTCTT CAG
```

IV. Second leader sequence.

```
  1 GTCCTTGTTG AGGAGGTACT CCTGATCGCT GTCCCAGTAC TTGGCGTGTG GGAAGCCGTC
 61 CTGATCG
```

V. Third leader sequence.

```
  1 CGATCCTCCT GCTGTTGCAG CGCTTCGGCA AACACGCGCA CCTGCTCTTC GGACCCGGCG
 61 AAGCGTTCGA CGAAGGCGTC TAGCCAGCAA CAGTCGCAAG
```

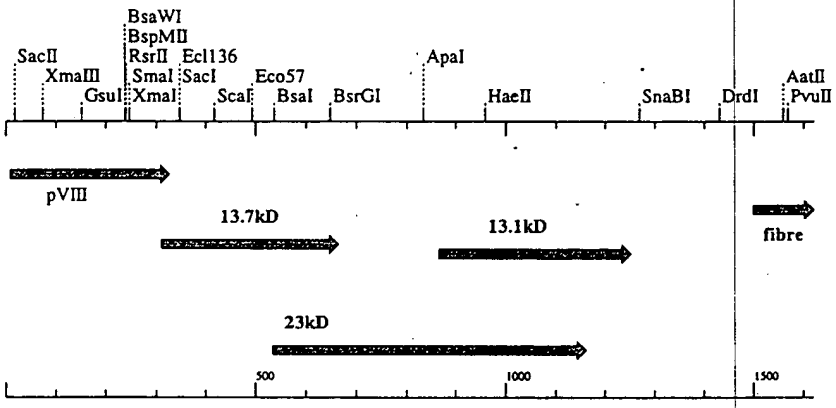
Figure 4

Sequence of the right hand end of the PAV genome this area being a proposed site for insertion of expression cassettes.

Nucleotide base count 183 A 255 C 306 G 204 T Total 948 bases

```
   1 CATCATCAAT AATATACCGC ACACTTTTAT TGCCCCTTTT GTGGCGTGGT GATTGGCGGA

  61 GAGGGTTGGG GGCGGCGGGC GGTGATTGGT GGAGAGGGGT GTGACGTAGC GTGGGAACGT

 121 GACGTCGCGT GGGAAAATAA CGTGGCGTGG GAACGGTCAA AGTCCGAGGG GCGGGGTCAA

 181 AGTCCGCAGT CGCGGGGCGG AGCCGGCTGG CGGGAATTCC CGGGACTTTC TGGGCGGGTA
                                          EcoRI  SmaI
 241 ATCGTTAACG CGGAGGCGGG GGAATTCCGA TCGGACGATG TGGTACTGAT TAACCGACCG
       HpaI                 EcoRI
 301 CAGGCGTGTC CACATCCGCT GTGGGTATAT CACCGGCGCT CGCGGTGTTC GCTCACACTC

 361 GTCTCGGCGC TGTCACAGAG AGAGACACTG AGAGCGAGAC GAGGAGAAAC CGAAAGCGGG

 421 GCAGGAGGAG TCAGCGGGCC ATCTTCCCAT CAGAGCCCTC TCATGGCCCA CGACCGACTG

 481 CTGCTGGCCG CGGTGGCTGA CTGTTGCTCG CCGTGCTCTA TCTGTACTTC GCCTACCTCG

 541 CGTGGCAGGA TCGGGACACT CTTCACACTC AGGAGGCCGC CTCTCCTCGC TTCTTCATCG

 601 GGTCCAACCA CCAGCCCTGG TGCCCGGATT TTGATTGGCA GGAGCAGGAC GAGCACACTC

 661 ACTAGACGTT TAGAAAAAAG ACACACATTG GAACTCATAT ATGTCTGCGG GACCGCATCA

 721 GCAGCCCGGT CTGCTGTTGG CTGCGGGTGA GAGGCCTCCG GTAATTCATC AGAACCGCAT
                                        StuI
 781 TCATCTGCGC CACGTCCCGA CATATGGTGC TGACGTCAGA ACAGCCCAGC GTGATCCTTT
                                      SacIII
 841 TAATGTGCTA GTCTACGTGC CCACTGGGTT TGCTGTGTTT GTGCCGACTG AGCGAGATTT

 901 TCAGAGGAGG GATCTGGTCC GTTTCCAGAC CTGCTGCTTC CGGCATCA
```

The Inverted Terminal Repeat (ITR) is shown in bold. Enzyme sites of interest are underlined with the enzyme name below. Putative TATA for E4 region is also shown.

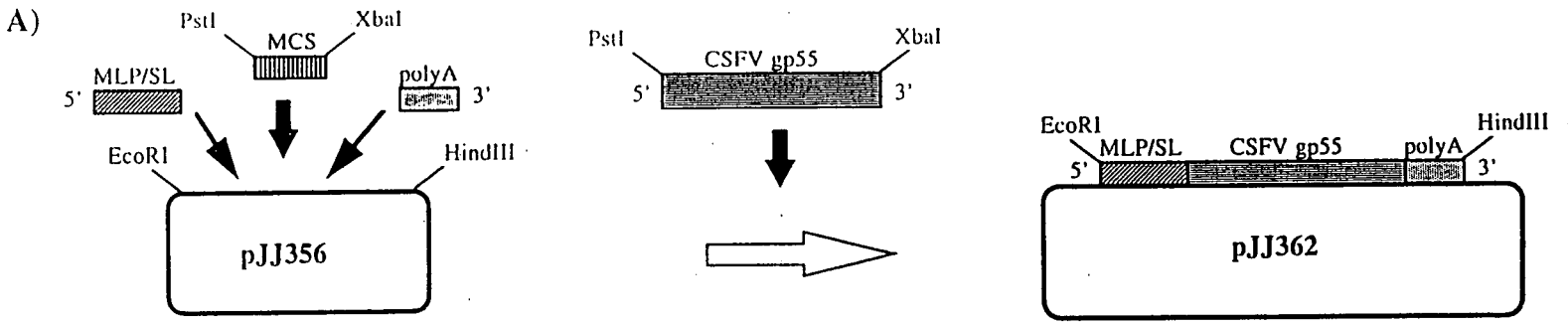


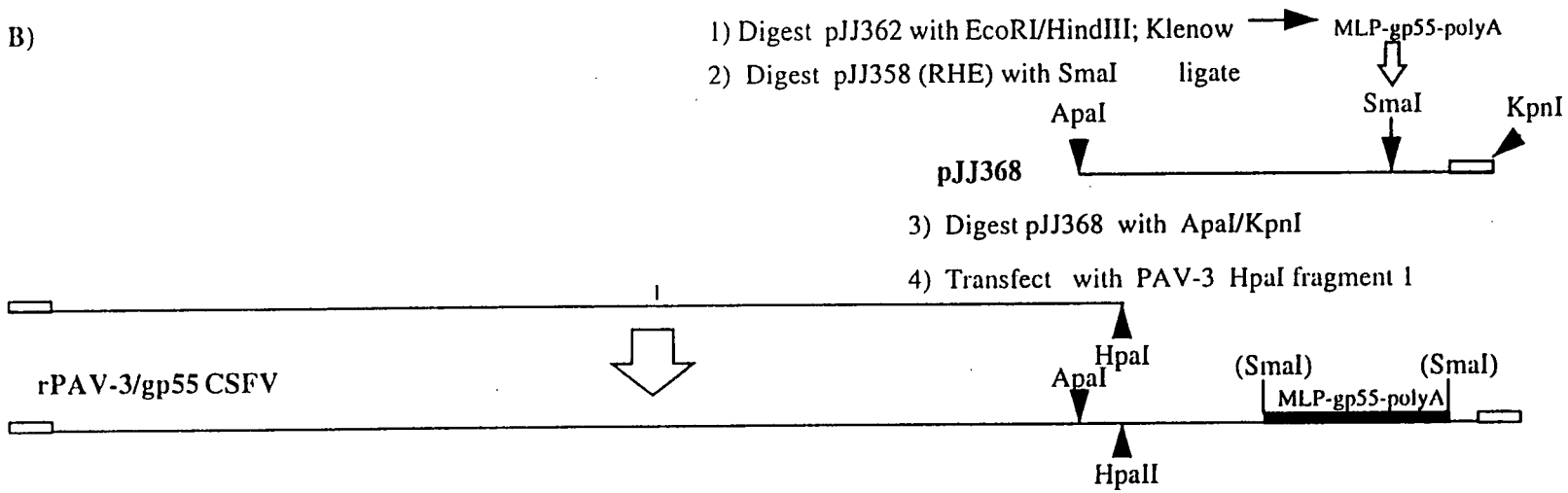
PAV3 E3 Sequence

(1618 bps)

Figure 5.

Figure 6





A) PAV-3 major late promoter and SV40 polyA sequences were cloned into pUC118 (pJJ356). Classical swine fever virus gp55 was cloned as a PstI/XbaI fragment into pJJ356 (pJJ362). PAV-3 right hand end fragment ApaI J was cloned into pUC118 in which the poly linker was changed to a ApaI-KpnI site (pJJ358).

B) Construction of the recombinant PAV-3: pJJ362 was digested with EcoRI/ HindIII, klenowed and blunt ligated into pJJ358 digested with SmaI (pJJ368). pJJ368 was digested with ApaI/KpnI and mixed with HpaI digested genomic PAV-3. The mix was transfected into primary pig kidney cells and a recombinant PAV-3 isolated (rPAV-3/gp55).

Figure 7. Temperatures of pigs after challenge with classical swine fever virus Weybridge

Pig#	Route	base temps	1*	2	3	4	5	6	7	8	9	10	11
4	s.c.	39.1	40.1	40.5	41.0	41.2	41.7	40.7	40.4	40.5	40.7	39.3	39.1 †
16	control	39.5	40.4	40.3	41.4	41.2	41.3	41.6 c,d	40.9 c,d	41.4 †			
24	control	39.5	40.6	40.7	41.3	41.2	41.7	41.5	40.7 c,†	-			

*: temperature day 1 post challenge
c: poor condition/wasting
d: diahorrea
†: euthanased
††: died

Figure 8.

Temperatures post challenge with CSFV